PRODUCTION OF 6–AMINOPENICILLANIC ACID THROUGH DOUBLE ENTRAPPED *ESCHERICHIA COLI* NCIM 2563

DEVENDER SINGH, REETA GOEL and B. N. JOHRI
Department of Microbiology, CBS & H, G.B. Pant University of Agriculture and Technology, Pantnagar 263 145, India.

ABSTRACT

Escherichia coli NCIM 2563 immobilized in agar-polyacrylamide by double entrapment methodology was found to convert penicillin G (benzyl penicillin) into 6–aminopenicillanic acid (6–APA). A level of 2.5% agar in these beads produced 10 mg/ml of 6–APA from 20 mg/ml benzyl penicillin. Penicillin acylase of this isolate was active at 37°C at pH 8. Cell suspension at the rate of 50 mg per 10 ml buffer gave the best yields. A batch culture cycle of 40 days was maintained with acylase activity dropping to 0.8 mg/ml of 6–APA at the X reuse. Dry immobilized cells stored at 40°C showed enzyme activity up to three months.

INTRODUCTION

There has been a rapid development of immobilized enzyme and cell technology for transformation of antibiotic molecules in the last decade. Production of 6–APA through immobilized cell system represents one such successful applications[1]. The formation of 6–APA is carried out by penicillin acylase which is produced by a variety of microorganisms including bacteria, actinomycetes and fungi[2]. The substrate spectrum of penicillin acylases is rather broad although they are considered as specific deacylating enzymes. A detailed account of the immobilization technology for 6–APA production exists which includes adsorption, cross-linking, covalent attachment and physical entrapment methodologies[3]. The purpose of this study is to develop a methodology which has a longer shelf life without loss in the production level.

MATERIALS AND METHODS

Organism and culture maintenance

Escherichia coli NCIM 2563 was maintained in nutrient agar slopes; working cultures were prepared by subculturing at 37°C for 24 h. For obtaining enzymatically active cells, *E. coli* was grown in a complex medium containing phenyl acetate[4].

Reaction mixture and assay for penicillin acylase

Cells were collected from the culture bath by centrifugation and stored at 4°C as suspension till further use. They were added to phosphate buffer (pH 8) containing benzyl penicillin (20 mg/ml). In continuous culture experiment, the antibiotic level was maintained at 5 mg/5 ml. The reaction mixture was incubated for 60 min at 37°C and 6–APA produced was estimated using *p*-dimethyl aminobenzaldehyde[5].

Immobilization methods

Four matrices were used for cell immobilization viz., calcium alginate, polyacrylamide, agar and agar + polyacrylamide for double entrapment. Alginate beads were prepared according to Kierstan and Bucke[6] using 50 mM phosphate buffer (pH 8). Polyacrylamide granules were obtained by suspending 10 ml cell suspension in 12.75 ml phosphate buffer containing 3 g acrylamide and 0.25 g of N, N'-methylene bis acrylamide. The catalytic system containing 2 ml of 2.5% potassium persulphate and 2.25 ml of 5%, N, N'N' tetramethyl ethylene diamine (TEMED) was rapidly added with continuous stirring[7].

Agar beads were prepared according to Kuo and Polack[8]. For double entrapment, agar beads were activated at 37°C for 24 h on a shaker. They were now placed in a solution of acrylamide (8%), bis acrylamide (0.5%) and TEMED (0.5%) in a nitrogen sparged container for 25 min. Beads were subsequently dropped into 0.5% potassium persulphate, left undisturbed for 3 min and then gently stirred for 30 min. Beads were subsequently washed with the buffer and air-dried.

RESULTS AND DISCUSSION

In the initial screening of the four carriers, alginate beads and polycrylamide granules produced only 35 to 50% 6–APA in the first cycle, which too dropped to a low level after II reuse; with agar beads 80%

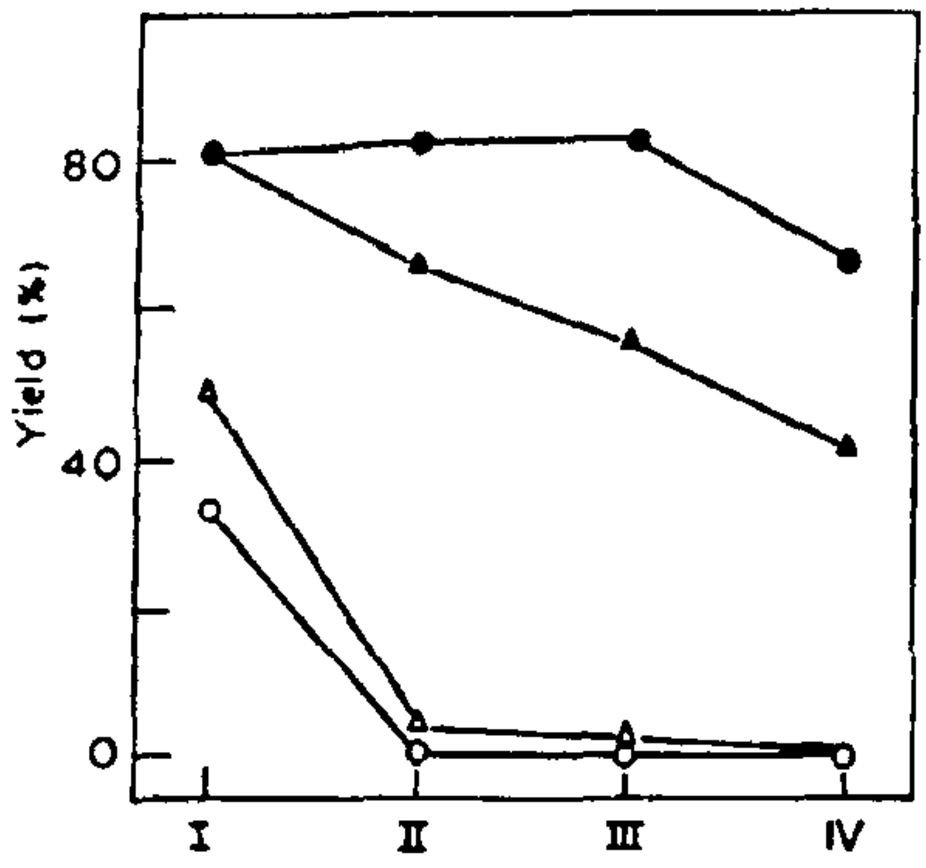

Figure 1. Screening of various matrices for 6–APA production using immobilized *E. coli* cells. [○, DE beads; ●, polyacrylamide granules; △, alginate beads; and ▲, agar beads.] Native cell activity is considered as 100% yield which was 12 mg/ml at 20 mg/ml of substrate concentration.

6–APA was estimated which dropped to only 50% after IV reuse. In the case of double entrapped beads there was a constant 6–APA level of over 80% for the first three cycles followed by a drop to about 65% at the 4th cycle (figure 1). In subsequent experiments, therefore, only the double entrapped beads were used and conditions favouring their optimum utilization were standardized. Inspite of remarkable success of alginate and polyacrylamide in other systems, it was not suitable due perhaps to the use of phosphate buffer, in which its stability is questionable[8,9].

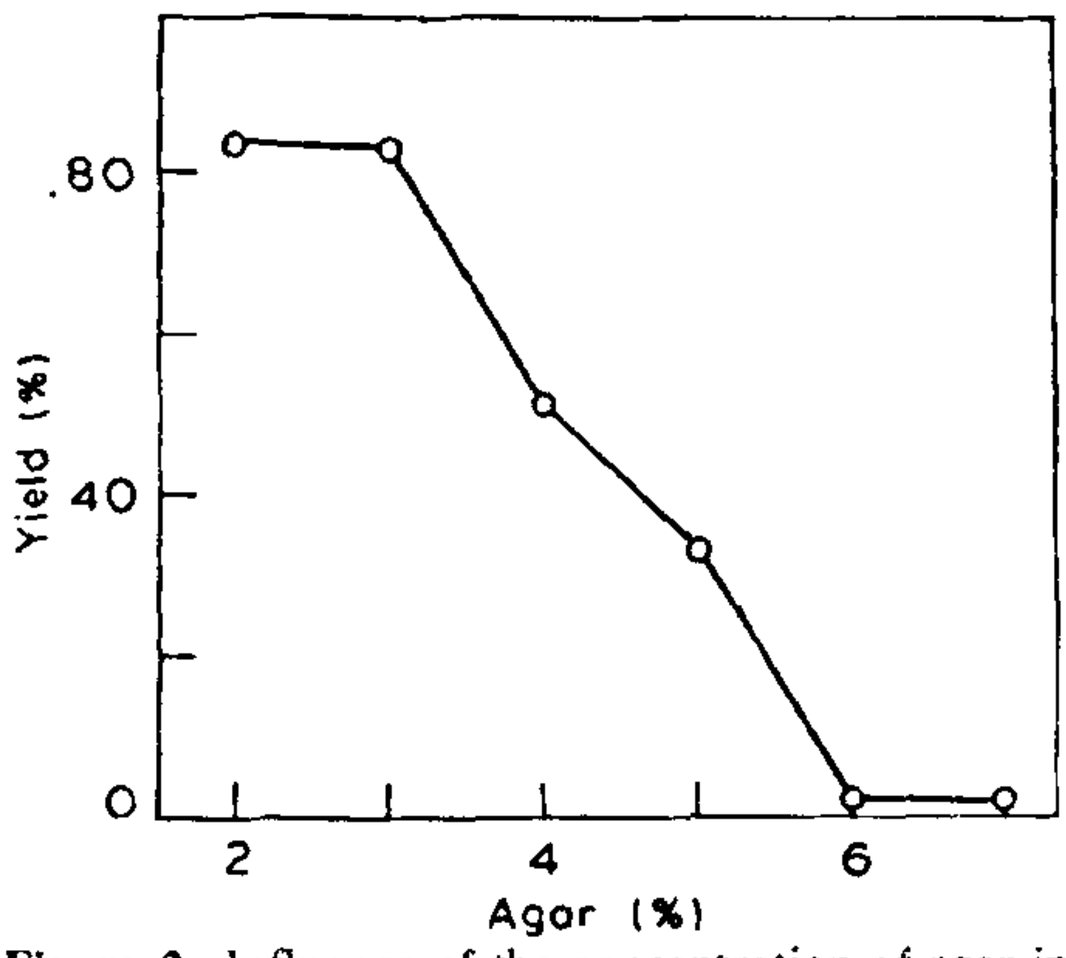

Figure 2. Influence of the concentration of agar in DE beads on the 6–APA yields. Native cell activity is considered as 100% yield.

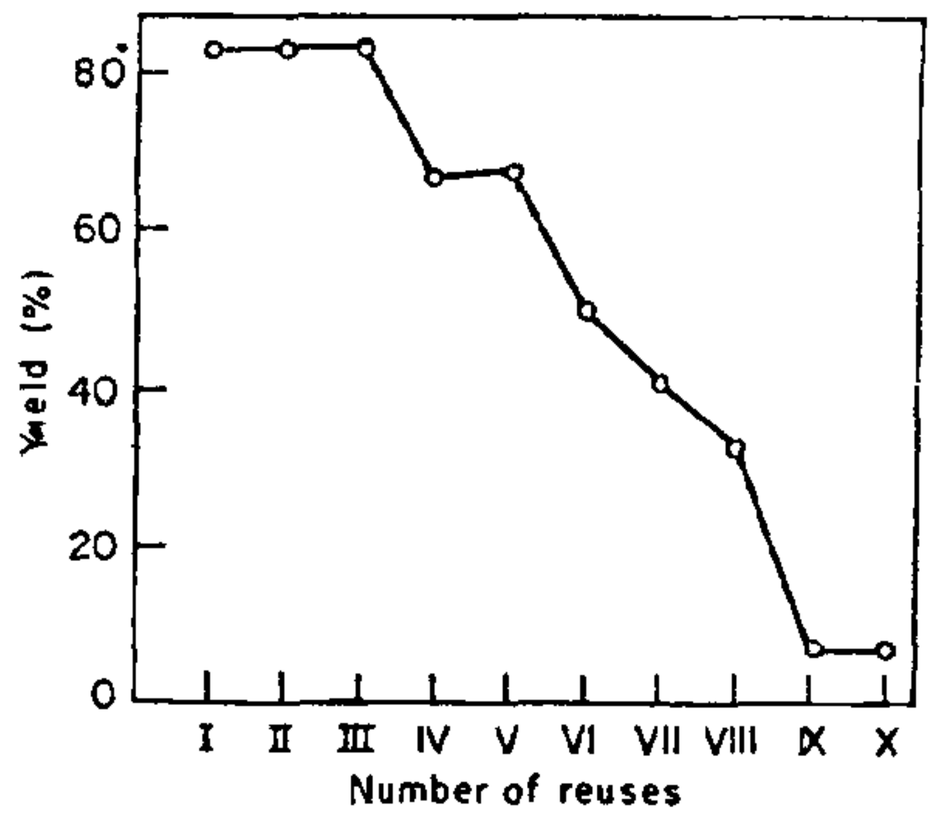

Figure 3. Reusability of DE beads for the production of 6–APA. Native cell activity is considered as 100% yield.

Double entrapped beads were able to bring about 50% conversion of benzyl penicillin at a cell level of 50 mg/10 ml in a final volume of 25 ml immobilized preparation. An agar concentration of 2.5% was optimum in the inner core (figure 2). The V_{max} for native and immobilized *E. coli* NCIM 2563 was 53.69 whereas K_m values were 30.87 and 36.24 mM. In the case of *E. coli* K–12, Cole[10] has reported a K_m of 0.02 mM for the pure enzyme and 30 mM for cell bound enzyme[10].

Beads were reused repeatedly up to ten times after gaps of 96 h inclusive of an activation period of 24 h; 6–APA production during this period declined from 10 to 0.8 mg/ml (figure 3). In a further test for reusability, the cells were dried at 45°C for 96 h and subsequently stored at 4°C. The yield of 6–APA from such beads after 60 and 90 days of storage was 6 and 5 mg/ml respectively. The substrate profile behaviour thus confirmed the reversible nature of the penicillin acylase[10].

In the case of immobilized system, binding of an enzyme or cell to a charged support is known to alter

Table 1 *Effect of pH and temperature on the release of 6–APA in DE beads*

pH	% yield*	Temp. (°C)	% yield*
3.5	0.3	25	1.0
5.0	1.7	30	50.0
6.5	21.2	35	83.3
8.0	83.3	40	66.6
9.5	50.0	45	8.3
11.0	8.3	50	1.6

*Native cell activity is considered as 100% yield which was 12 mg/ml at 20 mg/ml of substrate.

the pH activity profile and temperature shift[11]. Therefore, these were also examined for the double entrapped beads. An incubation time of 60 min supported maximum acylase activity at 37°C and pH 8 (table 1). In a batch process described by Lagerlof *et al*[3], the reaction temperature was controlled at 35°C and pH at 7.8. However, using fibre entrapped enzyme, Marconi *et al*[12] obtained the maximum yield at 37°C at pH 8. It would, therefore, appear that double entrapped beads provide a support which is comparable in efficiency to other systems in use.

To optimize the efficiency of double entrapped beads a continuous flow glass column was run with/without aeration at 39°C, pH 8 with a substrate concentration of 5 mg/ml in a total volume of 500 ml of phosphate buffer (figure 4). Reducing the reaction time (by increasing the flow rate) improved the conversion efficiency. In the case of aerated system 6-APA, yield declined from 0.15 to 0.034 mg/ml. Without aeration the drop in level was from 0.22 to 0.02 mg/ml. The flow rate under both the conditions was 3 and 0.5 ml/min. It is worth noting that the native cells produced 0.25 mg 6–APA/ml under similar conditions in batch culture. Good 6–APA yields have been reported when 5.5 of penicillin G was circulated through acylase fibres in a continuous system. However, 6–APA yield was 78% when epoxy cell immobilization methodology was utilized[13].

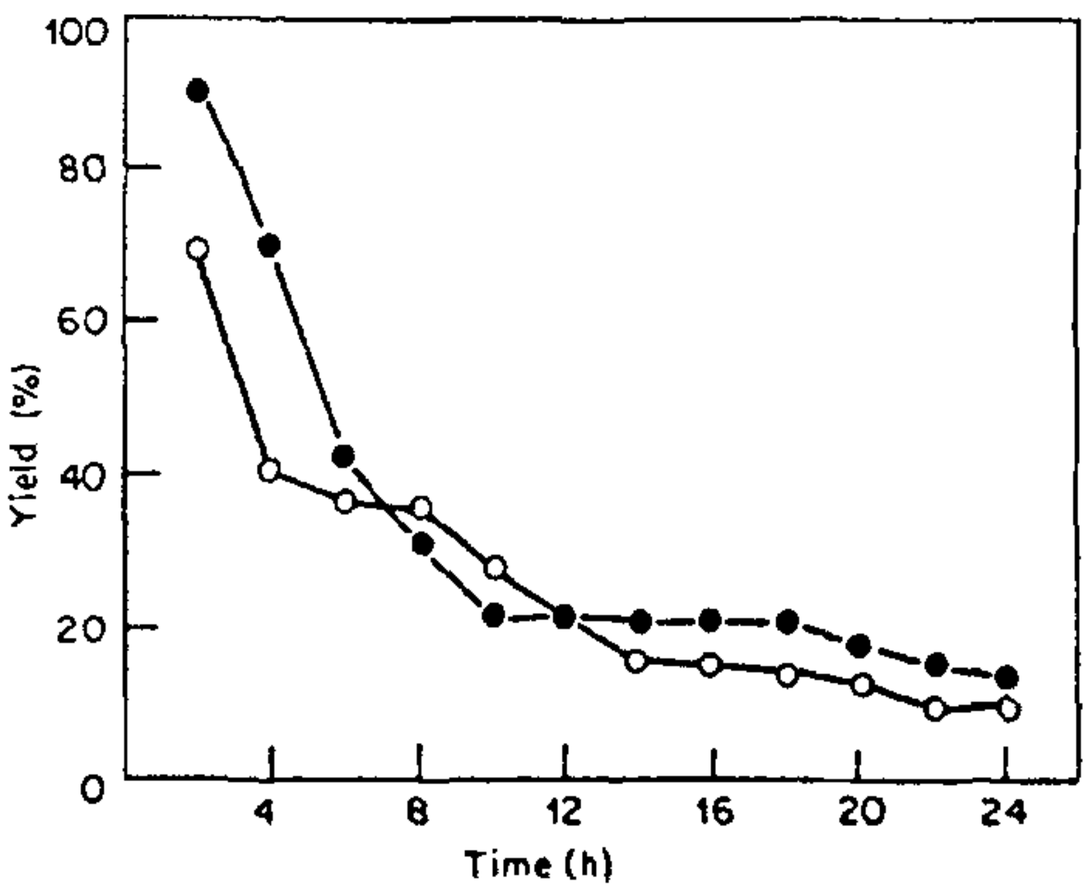

Figure 4. Production of 6–APA in a continuous flow reaction. [○, Column provided with aeration; ●, Column without aeration]. Native cell activity is considered as 100% yield which was 0.25 mg/ml at 5 mg/ml of substrate concentration.

It would be of interest to examine the double entrapped beads for large scale continuous column because there is sample scope in commercial sector.

5 December 1987: Revised 22 April 1968.

1. Vandamme, E. J., *Economic microbiology*, (ed.) A. H. Rose, Academic Press, New York, 1981, p. 467.
2. Claridge, C. A., Gourevitch, A. and Lein, J., *Nature (London)*, 1960, **187**, 237.
3. Lagerlof, E., Nathorst-Westfeld, L., Ekstorm, B. and Sjoberg, *Methods Enzymol.*, 1976, **44**, 759.
4. El-Saidd, H. M., Ei-Marsafy, M. K. and Darcosh, M. M., *Starke*, 1976, **28**, 282.
5. Balasingham, K., Warburton, D., Dunill, P. and Lilly, M. D., *Biochem. Biophys. Acta*, 1972, **276**, 250.
6. Kierstan, M. and Bucke, C., *Biotechnol. Bioeng.*, 1977, **19**, 387.
7. Mosbach, K. and Larsson, P. O., *Biotechnol. Bioeng.*, 1970, **12**, 19.
8. Kuo, W. Y. and Polack, J. A., *Biotechnol. Bioeng.*, 1983, **25**, 1985.
9. Kierstan, M., Darcy, G. and Reilly, J., *Biotechnol. Bioeng.*, 1982, **24**, 1507.
10. Cole, M., *Biochem. J.*, 1969, **115**, 733.
11. Kaufman, W. and Dauer, K., *Nature (London)*, 1964, **203**, 520.
12. Marconi, W., Morisi, F., Detta, P. C. and Roppuli, B. J., *Antibiotica*, 1973, **26**, 228.
13. Vandamme, E. J. (ed.), *Biotechnology of industrial enzymes*, Dekker, New York, 1984, p. 3.